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(57) Abstract

Transduction efficiencies of current viral vectors are inadequate to modify a sufficient number of tumor cells and achieve a relevant antitumor response. Replication of adenoviral vectors in tumor cells would amplify gene transduction. Adenoviral replication can be obtained in tumor cells by delivering genes essential for virus replication. Progeny virus are replication-defective, and the availability of replication-enabling genes limits the production of new cycles of replication. Cell viability determination showed that progeny virus specifically induced killing in GCV treated cells. A similar viral amplification effect was also shown in primary ovarian cancer cells derived from patients. Thus, this invention describes transcomplementation strategies to obtain replication of recombinant adenoviral vectors in ovarian tumor cells and in ovarian cancer primary cells.

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AMPLIFICATION OF GENE TRANSFER AND GENE THERAPY BY CONTROLLED REPLICATION

BACKGROUND OF THE INVENTION

Cross-Reference to Related Application

This application claims benefit of priority of U.S. provisional application Serial No. 60/076,812 filed March 3, 1998, now abandoned.

Federal Funding Legend

This invention was produced in part using funds obtained through grants RO1CA68245 and RO1CA72532 from the National Institutes of Health. Consequently, the federal government has certain rights in this invention.

Field of the Invention

The present invention relates generally to the field of gene therapy. More specifically, the present invention relates to a strategy by which to overcome limited tumor transduction and to enhance therapeutic effects.

Description of the Related Art

A variety of gene therapy strategies have been developed to

achieve antitumor effects. In this regard, molecular chemotherapy is an approach based on the selective delivery and expression of a gene-encoded toxin into cancer cells to achieve tumor eradication. The most common molecular chemotherapy system utilized to date to accomplish tumor cell killing has been the herpes simplex virus thymidine kinase (HSV-tk) gene given in combination with the prodrug ganciclovir (GCV) (1). However, other toxin gene systems have also been used in *in vitro* and *in vivo* models (2). Importantly, more than twenty clinical trials have been developed to exploit the toxin gene effects and achieve antitumor activity.

To date, the strategy of molecular chemotherapy has been mainly used in loco-regional disease models. In these *in situ* schemas, the toxin gene-encoding vector is administered intratumorally or into an anatomic compartment containing the tumor mass. The goals of this delivery method are to achieve high local vector concentration, to favor tumor transduction, and to limit vector dissemination. Transduction efficiencies of presently available vectors, however, have been shown to be suboptimal. Even in closed compartment delivery contexts, it has not been possible to modify a sufficient number of tumor cells to achieve a relevant tumoral response in clinical models (3-8). Furthermore, although transduction with HSV-tk followed by ganciclovir treatment can reduce tumor burden and prolong survival in various model systems, including those utilizing intratumoral and intraperitoneal administration, the required increased doses of viral vector needed for obtaining a quantitative tumor cell transduction is associated with limiting toxicity. In fact, substantial toxicity and experimental animal death has been noted (7, 9). Thus, the small therapeutic index of available vectors in the context of *in situ* administration is a critical

limiting factor for the purpose of gene therapy in the treatment of cancer.

Recombinant adenoviral vectors have been utilized for the direct *in situ* delivery of toxin genes into a variety of tumor localizations such as brain (22, 23), leptomeningeal cavity (24), head and neck (25), lung and pleural cavity (3, 5, 26), stomach (27), liver (9, 28, 29), prostate (30), peritoneal cavity and ovarian cancers (7, 21, 31, 32), and skin (33). The expression of toxin genes in these *in vivo* examples can reduce tumor burden and prolong survival in a variety of human epithelial cancer animal models when combined with prodrug administration (21).

With the molecular chemotherapy approach, there have not been frequent and durable complete tumor responses in clinically relevant circumstances. Tong et al. have argued that they achieved long-term survival in their nude mice inoculated with human ovarian tumors. The *sine qua non* for achievement of these, however, was a very low tumor burden and a very high dose of virus. In fact, 'cured' mice had been injected with the tumor tissue only 24 hours before receiving the viral treatment. Thus, their tumor burdens were, from the clinical standpoint, irrelevant. It was hypothesized that these limitations in the *in vivo* context were the result of inadequate tumor cell transduction. Attempts to address this limitation by simply increasing the administered viral dose have met with increasing, intolerable loco-regional toxicity (7, 9). To augment the percentage of cells transduced with a therapeutic gene, it was shown previously that tumor cells infected with a replication-defective recombinant adenovirus can be converted into recombinant adenovirus-producing cells by co-transduction with a replication-enabling plasmid (11, 12).

For these strategies of vector amplification to be

applicable *in vivo*, a certain level of restriction must be imposed in viral replication (37). Kucharczuk et al. have recently shown that a mutant herpes virus can reduce tumor burden and prolong survival in an intraperitoneal model of human mesothelioma (38). The virus contains a mutation that attenuates its ability to replicate in normal cells, but does not appear to affect its replication in rapidly dividing cells. After intraperitoneal viral administration in SCID mice, the authors could observe a prolonged survival, without detecting viral dissemination. This system, however, has notable limitations. The basis for the attenuation of viral replication in normal tissues with this mutant virus is still unclear, cell susceptibility to herpes lysis *in vitro* is not universal, and the viral treatment produced an unexplained early high rate of animal death. Thus, a more complete understanding of that vector is warranted before further consideration is given to its clinical potential.

Recently, McCormick et al. reported the development of a mutated recombinant adenovirus exhibiting selective replication in cells lacking a functional p53 gene. In this regard, wild-type adenoviruses induce cells to enter into S phase of the cell cycle to allow for replication of its own viral DNA. Entering into S phase requires the inactivation of the cellular protein p53, a task that normally is accomplished by the adenoviral E1B protein. It was hypothesized that mutant adenoviruses lacking E1B would be unable to replicate in cells expressing functional p53, but would otherwise replicate in p53-deficient cells (39). Because p53 is absent in many tumors, a selective replicative system based on this lytic virus was proposed for cancer therapy purposes. However, extensive studies in a variety of cell lines and animal tumor models have failed to confirm the selective properties of the virus to replicate only in p53 mutant tumor cells. Thus, the use of replicative systems for

obtaining vector-mediated oncolysis, although a promising strategy for cancer treatment, has met with toxicity and specificity problems.

Despite the limitations already mentioned, replicative systems based on recombinant adenoviruses have some *a priori* advantages, which are as follows: 1) the viral life cycle is lytic, 2) there is an established record of safety, including the historical use of adenoviral vaccines and the presence of widespread and 'benign' infections in the population, 3) molecular characterization of the virus is extensive, 4) production is relatively easy, with high viral titers readily obtainable, 5) there is no latency in the viral life cycle, and 6) it has no tropism for the nervous system.

Thus, the prior art is deficient in successful transduction and targeting of adenoviral vector(s) to specific tissues and/or tumors without resulting in toxicity to surrounding tissues or organs. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

One means to potentially circumvent current obstacles encountered in gene therapy is to employ a vector that can be locally amplified after tumor cell transduction (10). Controlled replication of the delivered viral vector is necessary to achieve this amplification effect. To that end, viral replication can be restricted to the targeted tumor cells by limiting the expression of genes essential for viral replication only to the tumor cells of interest. Progeny virus from transduced tumor cells would lead to 1) infection of neighboring tumor cells, 2) increased local viral inoculum, and 3) augmentation of

therapeutic gene expression. In this regard, it has been shown that simultaneous addition of an E1-deleted, replication-incompetent adenovirus and a replication-enabling plasmid in a trans orientation could convert the co-transduced cells into recombinant adenovirus-producing cells (11, 12). Such a phenomenon could be exploited to achieve high local concentration of viral vectors, specifically within the tumor. This spatially restricted increase in viral inoculum augments transgene expression without enhancing regional toxicity, thus effecting a more favorable therapeutic index.

To determine the feasibility of increasing the viral inoculum by controlled replication in a closed compartment, an ovarian cancer model system was chosen. The present invention demonstrates that simultaneous addition into ovarian cancer cell lines of a replication incompetent E1-deleted adenovirus expressing HSV-tk (AdCMVHSV-tk) and plasmids providing the E1 sequences needed for replication results in the production of functional antitumoral adenovirus. Importantly, this effect can be reproduced in primary ovarian cancer cells derived from patients. Such an amplification strategy may, thus, offer the means to circumvent the limitations of current approaches, whereby suboptimal tumor transduction hinders the utility of molecular chemotherapy.

One object of the present invention is to provide a viral amplification composition, comprising a replication-incompetent, recombinant adenoviral vector, wherein the vector contains a therapeutic gene and at least one gene encoding a replication-enabling function for a non-adenoviral vector. The therapeutic gene can be selected from the group consisting of the herpes simplex virus thymidine kinase gene, the cytosine deaminase gene and the purine nucleoside phosphorylase gene and the gene encoding the non-adenoviral

vector replication-enabling function is a gene encoding a necessary viral packaging protein. The recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting of E1, E2 and E4; and a replication-incompetent, non-adenoviral vector, wherein the vector contains at least one gene encoding an adenoviral replication-enabling function, wherein the gene encoding adenoviral replication-enabling function is selected from the group consisting of E1A, E1B, E2 and E4, wherein the non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding a necessary viral packaging protein(s). The non-adenoviral vector can be selected from the group consisting of adeno-associated viral vectors and RNA viral vectors, wherein the RNA viral vector may be an alphavirus and the RNA viral vector may be a Sindbis virus, wherein following transduction of a cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions, wherein mutual transcomplementation establishes replication competance and results in controlled viral amplification in the cell and neighboring cells.

In an embodiment of the present invention, there is provided a method by which targeted expression of a therapeutic gene is achieved by controlled viral amplification, comprising the steps of co-transducing a cell with (a) a replication-incompetent, recombinant adenoviral vector containing a therapeutic gene and at least one gene encoding a replication-enabling function for a non-adenoviral vector, wherein the recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting

of E1, E2 and E4. The gene encoding said non-adenoviral vector replication-enabling function may be a gene encoding a necessary viral packaging protein and the therapeutic gene is selected from the group consisting of the herpes simplex virus thymidine kinase gene, the cytosine deaminase gene and the purine nucleoside phosphorylase gene; and (b) a replication-incompetent, non-adenoviral vector containing at least one gene encoding adenoviral replication-enabling functions, wherein the non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding necessary viral packaging protein(s), wherein the genes encoding adenoviral replication-enabling functions are selected from the group consisting of E1A, E1B, E2 and E4, wherein following transduction of a target cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions and mutual transcomplementation establishes replication competance resulting in controlled viral amplification in the target cell and neighboring cells and the therapeutic gene is expressed in the target cell, wherein the cells are selected from the group consisting of ovarian cancer cells and colon cancer cells.

In yet another embodiment of the present invention, a method is provided by which tumor cells are killed, comprising the steps of (1) co-transducing said tumor cell with (a) a replication-incompetent, recombinant adenoviral vector containing the herpes simplex virus thymidine kinase gene and at least one gene encoding replication-enabling functions for a non-adenoviral vector. The recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting of E1, E2 and E4.

The gene encoding said non-adenoviral vector replication-enabling function is a gene encoding a necessary viral packaging protein; and (b) a replication-incompetent, non-adenoviral vector containing at least one gene encoding adenoviral replication-enabling functions, wherein the non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding a necessary viral packaging protein(s), wherein said gene encoding said adenoviral replication-enabling function is selected from the group consisting of E1A, E1B, E2 and E4, wherein said non-adenoviral vector is selected from the group consisting of adeno-associated viral vectors and RNA viral vectors, wherein following transduction of the tumor cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions establishing replication competance resulting in controlled viral amplification in the tumor cell and neighboring cells; and (2) treating the co-transduced tumor cells with ganciclovir, wherein ganciclovir is converted to a toxin by the product of the thymidine kinase gene resulting in tumor cell cytotoxicity, wherein the tumor cells are selected from the group consisting of ovarian cancer cells and colon cancer cells.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present

specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

Figure 1 shows a schema of the transcomplementation strategy of the present invention. An example of *in situ* viral amplification by mutual transcomplementation based on recombinant adenovirus and Sindbis viruses.

Figure 2 shows the production of progeny recombinant adenovirus in a panel of ovarian cancer cell lines. Viral titers (pfu/ml) were determined by plaque assays of cell lysates obtained 48 hr after co-transduction of cells with AdCMVHSV-tk/pL and the adenoviral replication-enabling sequences E1A and E1B. Control groups received virus only or the control plasmid pcDNA3. In addition, HeLa cells, highly transducible by adenovirus-polylysine conjugates, were included in the experiments as a control for the co-transduction efficiency.

Figure 3 shows the transduction efficiency with AdCMVHSV-tk/pL in a panel of ovarian cancer cell lines. Luciferase expression (RLU/mg of total protein) was determined 24 hr after co-transduction of cell lines with AdCMVHSV-tk/pL and pCMVLuc. Each histogram represents the mean average and standard deviation of duplicate experiments.

Figure 4 shows the functionality of progeny AdCMVHSV-tk adenovirus after infection and treatment of SKOV3.ip1. Cell lysates obtained by co-transduction of AdCMVHSV-tk/pL and replication-enabling sequences were plaque titered. Infection of SKOV3.ip1 cells was performed in a 96-well plate, in triplicate wells, with a multiplicity of infection of 1 plaque forming unit (pfu)/cell. To control for the

cytotoxic effect of recombinant adenovirus itself, a second experimental group was infected with an adenovirus encoding the firefly luciferase reporter gene. After 24 hr, GCV was added to half of the samples in both groups. Cell survival was determined after 5 days of treatment by means of a colorimetric cell proliferation assay. Two independent experiments were performed. Bars, mean absorbance values obtained in a representative experiment, ± SD of the mean.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "replication-enabling" shall refer to genes or proteins that provide the required functions for viral replication to occur.

As used herein, "replication-incompetent" shall refer to a virus or vector that, by virtue of gene deletions in its genome, is incapable by itself of replication.

As used herein, "viral amplification" shall refer to the multiplication and resultant increase in the number of virus particles following replication.

As used herein, "mutual transcomplementation" shall refer to the process by which both viruses provide to each other, in the cellular context, the required genetic functions for replication.

As used herein, "non-adenoviral vector" shall refer to any vector that is not an adenoviral vector, i.e., a "non-adenoviral vector" is a vector not substantially comprised of adenoviral sequences. "Non-adenoviral vectors" may include adeno-associated viral vectors, RNA viral vectors, plasmids, etc.

The strategy of the present invention, based on the co-

delivery of replication-enabling sequences with both the recombinant adenovirus and another vector, has an additional specific advantage. Clinical development of replicative viral vectors has been hindered by safety considerations, including fear of uncontrolled replication in normal tissues and collateral damage. In this regard, replicative vector systems based on two components (two viruses, or one virus and a plasmid or protein, etc.) offer the advantage of being intrinsically controllable at two different levels. First, progeny adenoviruses are themselves replication-incompetent. This fact implies that productive replication cycles can only continue in the particular region where replication is taking place if, and only if, there remains cells that are both transducible by adenovirus and that continue to express replication-enabling sequences. These conditions can be dependent on therapeutic maneuvers, i.e., successive (cycled) administration of required vectors. Second, the replication-enabling sequence can be engineered to contain regulatory elements that limit the expression of its genes, essential for viral replication, to certain targeted tissues or tumor cell types. Rodriguez et al. have recently constructed a replicative adenovirus with the viral gene E1A under the control of prostate-specific antigen (PSA), and showed that its replication is restricted primarily to PSA-producing cells (40). Thus, the administration of replication-enabling sequences by means of a second vector may offer the possibility of commanding, to a certain level, the production of vector replication cycles.

The present invention is directed towards a viral amplification composition, comprising a replication-incompetent, recombinant adenoviral vector. The vector contains a therapeutic gene and at least one gene encoding a replication-enabling function for a

non-adenoviral vector. The therapeutic gene may be selected from the group consisting of the herpes simplex virus thymidine kinase gene, the cytosine deaminase gene and the purine nucleoside phosphorylase gene and the gene encoding said non-adenoviral vector replication-enabling function may be a gene encoding a necessary viral packaging protein, wherein the recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting of E1, E2 and E4; and a replication-incompetent, non-adenoviral vector. The vector contains at least one gene encoding an adenoviral replication-enabling function and the gene encoding adenoviral replication-enabling function is selected from the group consisting of E1A, E1B, E2 and E4. The non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding a necessary viral packaging protein(s), and non-adenoviral vector is selected from the group consisting of adeno-associated viral vectors and RNA viral vectors. The RNA viral vector may be an alphavirus and RNA viral vector may be a Sindbis virus, wherein following transduction of a cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions, wherein mutual transcomplementation establishes replication competance and results in controlled viral amplification in the cell and neighboring cells.

The present invention also provides a method by which targeted expression of a therapeutic gene is achieved by controlled viral amplification, comprising the steps of co-transducing a cell with (a) a replication-incompetent, recombinant adenoviral vector containing a therapeutic gene and at least one gene encoding a replication-

enabling function for a non-adenoviral vector, wherein the recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting of E1, E2 and E4. Preferably, the gene encoding said non-adenoviral vector replication-enabling function is a gene encoding a necessary viral packaging protein and the therapeutic gene is selected from the group consisting of the herpes simplex virus thymidine kinase gene, the cytosine deaminase gene and the purine nucleoside phosphorylase gene; and (b) a replication-incompetent, non-adenoviral vector containing at least one gene encoding adenoviral replication-enabling functions, wherein said non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding necessary viral packaging protein(s). Preferably, the genes encoding adenoviral replication-enabling functions are selected from the group consisting of E1A, E1B, E2 and E4, wherein following transduction of a target cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions and mutual transcomplementation establishes replication competance resulting in controlled viral amplification in the target cell and neighboring cells and the therapeutic gene is expressed in the target cell, wherein said cells are selected from the group consisting of ovarian cancer cells and colon cancer cells.

The present invention further provides a method by which tumor cells are killed, comprising the steps of (1) co-transducing a tumor cell with (a) a replication-incompetent, recombinant adenoviral vector containing the herpes simplex virus thymidine kinase gene and at least one gene encoding replication- enabling functions for a non-

adenoviral vector. Preferably, the recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting of E1, E2 and E4. Preferably, the gene encoding said non-adenoviral vector replication-enabling function is a gene encoding a necessary viral packaging protein. Secondly, a replication-incompetent, non-adenoviral vector containing at least one gene encoding adenoviral replication-enabling functions, wherein the non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding a necessary viral packaging protein(s). Preferably, the gene encoding said adenoviral replication-enabling function is selected from the group consisting of E1A, E1B, E2 and E4. Preferably, the non-adenoviral vector is selected from the group consisting of adeno-associated viral vectors and RNA viral vectors. Following transduction of the tumor cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions establishing replication competance resulting in controlled viral amplification in the tumor cell and neighboring cells. Subsequently, the co-transduced tumor cells are treated with ganciclovir, wherein ganciclovir is converted to a toxin by the product of said thymidine kinase gene resulting in tumor cell cytotoxicity. Representative examples of tumor cells treatable with this method include ovarian cancer cells and colon cancer cells.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch

& Sambrook, "Molecular Cloning: A Laboratory Manual (1982), "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985), "Oligonucleotide Synthesis" (M.J. Gait ed. 1984), "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)], "Transcription and Translation" [B.D. Hames & S.J. Higgins eds. (1984)], "Animal Cell Culture" [R.I. Freshney, ed. (1986)], "Immobilized Cells And Enzymes" [IRL Press, (1986)], B. Perbal, "A Practical Guide To Molecular Cloning" (1984). Therefore, if appearing herein, the following terms shall have the definitions set out below.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form or double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, i.e., capable of replication under its own control. An "origin of replication" or "replication-enabling functions" refer to those DNA sequences that participate in replicative DNA synthesis. "Replication- incompetency" would hence be a

consequence of the interruption (i.e. due to recombination, manipulation using standard molecular biology techniques, etc.) or loss (i.e. due to genetic deletion) of these DNA sequences, and "replication competency" would be restored after complementation of the mutated gene(s). An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "operably linked" and "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

In general, expression vectors containing promoter sequences which facilitate the efficient transcription and translation of the inserted DNA fragment are used in connection with the host. The expression vector typically contains an origin of replication, promoter(s), terminator(s), as well as specific genes which are capable of providing phenotypic selection in transformed cells. The transformed hosts can be fermented and cultured according to means known in the art to achieve optimal cell growth.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence. A "cDNA"

is defined as copy-DNA or complementary-DNA, and is a product of a reverse transcription reaction from an mRNA transcript.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell. A "cis-element" is a nucleotide sequence, also termed a "consensus sequence" or "motif", that interacts with other proteins which can upregulate or downregulate expression of a specicif gene locus. A "signal sequence" can also be included with the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell and directs the polypeptide to the appropriate cellular location. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. The promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

The term "oligonucleotide" is defined as a molecule comprised of two or more deoxyribonucleotides, preferably

more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide. The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to enzymes which cut double-stranded DNA at or near a specific nucleotide sequence.

"Recombinant DNA technology" refers to techniques for uniting two heterologous DNA molecules, usually as a result of *in vitro* ligation of DNAs from different organisms. The resulting molecule is often termed a "recombinant". Recombinant DNA molecules are commonly produced by experiments in genetic engineering. Synonymous terms include "gene splicing", "molecular cloning" and "genetic engineering". The product of these manipulations results in a "recombinant" or "recombinant molecule".

A cell has been "transformed", "transfected" or "transduced" with exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a vector or plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations. An organism, such as a plant or animal, that has been transformed with exogenous DNA is termed "transgenic".

It is specifically contemplated that pharmaceutical compositions may be prepared using the vectors of the present invention. In such a case, the pharmaceutical composition comprises the vectors of the present invention and a pharmaceutically acceptable carrier. A person having ordinary skill in this art would readily be able to determine, without undue experimentation, the appropriate dosages and routes of administration of thess vectors of the present invention. When used for *in vivo* for therapy, the vector of the present invention is administered to the patient or an animal in therapeutically effective amounts, i.e., amounts that eliminate or reduce the tumor burden. It will normally be administered parenterally, preferably intravenously, but other routes of administration will be used as appropriate. The dose and dosage

regimen will depend upon the nature of the cancer (primary or metastatic) and its population, the characteristics of the particular immunotoxin, *e.g.*, its therapeutic index, the patient, the patient's history and other factors. The amount of vector administered will typically be in the range of about 0.01 mg/kg to about 100 mg/kg of patient weight. The schedule will be continued to optimize effectiveness while balanced against negative effects of treatment. *See* Remington's Pharmaceutical Science, 17th Ed. (1990) Mark Publishing Co., Easton, Penn., and *Goodman and Gilman's: The Pharmacological Basis of Therapeutics* 8th Ed (1990) Pergamon Press, which are incorporated herein by reference. For parenteral administration, the vectors will most typically be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are preferably non–toxic and non–therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, *e.g.*, buffers and preservatives.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Experimental strategy of mutual transcomplementation

The components of this process are replication-

defective viral vectors that encode and mutually provide to each other, in cells that have been transduced with both, the required genetic sequences for establishing replication competence. As a consequence, both vectors replicate and spread to nearby cells, where this process continues in a cascading manner by virtue of the expression of replication-enabling sequences. This replicative vector system offers the advantage of being intrinsically controllable. The basis for control resides in three factors. First, viral replication depends on the presence of all the component vectors, which can be controlled by the timing of vector administration. Second, the expression of at least one of the component vectors is transient in nature, thus limiting the time during which required elements coexist in transduced cells. Third, progeny virus themselves are replication-defective.

Controlled viral replication could be therapeutically useful by three mechanisms. First, production of progeny virus from the transduced cells would allow a local increase in therapeutic virus inoculum and infection of neighboring cells, thus augmenting delivery of therapeutic transgenes. Second, and as a derivation of the former, augmentation of mass of viruses, in the context of immunopotentiation strategies, can provide for the required immunogenic mass to provoke an effective immune response. Third, the use of viruses displaying a lytic life cycle would allow virus-mediated tumor cell lysis.

Figure 1 shows a schema of the mutual transcomplementation strategy of the present invention. In this example, a DNA adenovirus is cotransduced with an RNA alphavirus (Sindbis). The use of an RNA vector as one of the components of the system avoids the possibility of genetic recombination of the replication-enabling sequences with the genome of the other vector

component. However, mutual transcomplementation can also be achieved with adenovirus and adeno-associated virus components.

EXAMPLE 2

Cell lines and measurement of new virus production

The human ovarian carcinoma cell lines PA-1 and SW626, and the human cervical carcinoma cell line HeLa, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The human ovarian carcinoma cell lines OV4 and SKOV3.ip1 were provided by Timothy J. Eberlein (Brigham and Women's Hospital, Harvard Medical School, Boston, MA), and Janet Price (Baylor University, Houston, TX), respectively. These cell lines were maintained in Dulbecco's Modified Eagle's Medium/F12 (Mediatech, Herndon, VA) supplemented with L-glutamine (300 µg/ml), penicillin (100 I.U./ml), streptomycin (25 µg/ml) and 10% heat-inactivated fetal calf serum (Hyclone, Logan, Utah) at 37°C in a humidified, 5% CO_2 atmosphere. The human ovarian carcinoma cell line OVCAR-3 was provided by Donald Buschbaum (University of Alabama at Birmingham, Birmingham, AL) and maintained in RPMI 1640 media (Mediatech, Herndon, VA) supplemented with L-glutamine, penicillin, streptomycin, 20% heat-inactivated fetal calf serum and 10 µg/ml bovine insulin (Gibco Life Technologies, Gaithersburg, MD), under the same conditions. Primary cells were obtained from human ascites fluid from patients with cytomorphologically proven ovarian carcinoma at the time of laparotomy or paracentesis (13).

Infectious adenoviral particles present in supernatants and cell lysates of human ovarian cancer cells co-transduced with

adenovirus and pUC-E1A and pUC-E1B plasmids were quantified by counting pfu's in titering assays done with permissive 293 cells. In these assays, infections were performed in 6-well plates with 100 µL of diluted virus for 1 hour, followed by two washes of the cells with PBS. Reading of the assays was performed on day 14 after infection.

EXAMPLE 3

Production of adenovirus-polylysine conjugates

The highly efficient adenovirus-polylysine (AdpL) molecular conjugate vector system was used as a means to deliver replication-enabling plasmids along with the functional virus *in vitro* employing methods described (14-17). Adenovirus-polylysine conjugates are composed of a DNA binding domain based on the polycation polylysine and an endosomolytic domain based on a replication-incompetent adenovirus (15). In the current invention, the adenovirus had a third function, it encoded the conditionally cytotoxic HSV-tk gene. In this regard, an E1-deleted, replication defective adenoviral vector encoding the herpes simplex thymidine kinase gene, AdCMVHSV-tk, was employed.

Serotype 5 adenovirus AdCMVHSV-tk, constructed previously (13), was amplified in permissive 293 cells. Cellular lysates were made by freeze-thawing two days post-infection and virus particles were isolated by two cesium chloride (CsCl) gradient clarifications. The viral solution was resuspended in CsCl density 1.33 to a final volume of 2.5 ml and loaded in a PD10 column (Pharmacia, Piscataway, NJ) pre- equilibrated with HEPES buffered

saline (HBS: 20 mM HEPES (pH 7.8), 150 mM NaCl). After elution with 2 ml of HBS, virus was resuspended to a final volume of 3.6 ml with HBS and then incubated on ice for 4 hr with 0.4 ml poly-L-lysine (Sigma, St. Louis, MO) and 10 mg of 1-ethyl-3-(dimethylaminopropyl) carbodimide hydrochloride (EDC) in 0.04 ml of distilled, deionized water (Pierce Biochemicals, Rockford, IL). Viruses were isolated by a subsequent cesium chloride gradient centrifugation and diluted with an equal volume of viral preservation media (50% glycerol, 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mg/ml bovine serum albumin). The number of viral particles was estimated by spectroscopy at 260 nM, in which 1 O.D.=1 x 10^{12} viral particles per ml (18). Conjugated virus, referred to as AdCMVHSV-tk/pL, were diluted with viral preservation media to achieve a concentration of 1 x 10^{11} particles/ml and stored in aliquots at -70°C until further use.

EXAMPLE 4

Construction of replication-enabling plasmids

Plasmids were constructed to provide the region of the adenoviral genome that is absent in E1-deleted, replication-incompetent adenovirus, as described (19). Briefly, adenovirus E1A and E1B sequences separated on two plasmids, designated pUC-E1A and pUC-E1B, were used to reduce the possibility of homologous recombination of replication-enabling genes within the adenovirus genome when co-transduced with E1-defective adenovirus. Both components of the E1 region were under the transcriptional control of the active viral promoter elements. A plasmid expressing the luciferase reporter gene, pCMVluc4, provided by M. Cotten, (Institute of Molecular

Pathology, Vienna, Austria), was used as a negative control. All plasmids were isolated according to endotoxin-free procedures with a commercial kit (EndoFree Plasmid Maxi Kit, Qiagen, Chatsworth, CA).

EXAMPLE 5

Co-transduction of adenoviruses and plasmids

Co-transduction of the AdCMVHSV-tk virus and the replication-enabling plasmids, pUC-E1A and pUC-E1B, was accomplished by an ionic linkage between the virus and plasmid DNA, as described (20). Briefly, 100 μL of the adenovirus-polylysine conjugate AdCMVHSV-tk/pL was incubated with 3.0 μg each of pUC-E1A and pUC-E1B plasmids in 200 μL of HEPES buffered saline (HBS) for 30 minutes at room temperature. Then, 4 μL of a 1 μg/μL distilled water stock solution of free polylysine was diluted in 300 μL of HBS, and the mixture added to the DNA/adenovirus mix. The polylysine was permitted to complex further with the DNA/adenovirus mix for an additional 30-min period at room temperature. Control complexes were made simultaneously by substituting control plasmids pCMVluc4 or pcDNA3 (Invitrogen, San Diego, CA) for the replication-enabling plasmids.

To determine transduction efficiency with the adenovirus-polylysine conjugate in ovarian cancer cells, both pCMVluc4 and the plasmid pCMVgal, expressing β-galactosidase (from Grant MacGregor, Howard Hughes Medical Institute, Houston, TX) were used as reporter gene plasmids. Reporter gene expression assays were performed according to standard techniques (13). For the *in vitro* studies, human ovarian carcinoma cells were plated (5×10^5 cell/well) in 6-well plates

18 hr before transduction. For transduction, each well received 100 µL of complex in 1 ml of reduced serum media (Opti-MEM, Gibco Life Technologies, Gaithersburg, MD) for 60 min at 37°C. Cells were then washed three times with phosphate buffered saline (pH 7.3) (1X PBS) to eliminate unbound virus and 2 ml of media containing 10% serum was added. Fourty-eight hours after transduction, cells were harvested and stored at -70°C.

EXAMPLE 6

Toxin gene killing experiments

To functionally validate the virus progeny obtained in co-transduced ovarian cancer cells, *in vitro* toxin gene cell killing assays were performed. Cell lysates were obtained in co-transduction experiments, titered as described above, and used for infecting SKOV3.ip1 cells. Cells were plated in triplicate wells on 96 well plates, at 5,000 cells per well, and infected with 1 plaque forming unit (pfu)/cell. In parallel, cells were also infected with an control adenovirus encoding the firefly luciferase reporter gene, AdCMVLuc (provided by R. Gerard, University of Texas-Southwestern Medical Center, Dallas, TX). Half of the samples were treated 24 hours later with 20 µM GCV (Cytovene, Syntex Laboratories, Inc., Palo Alto, CA). Cell viability was determined after 7 days using a colorimetric cell proliferation assay that measured conversion of a tetrazolium salt to formazan by viable cells as described by the manufacturer (Cell Titer 96 Aqueous Non-radioactive Cell Proliferation Assay, Promega, Madison, WI). Briefly, 20 µL assay

mixture was added to each cell well and the plates were incubated for 3 hours at 37°C before absorbance was measured at 490 nm in a 96 well plate reader (Molecular Devices, Menlo Park, CA). To quantify cell numbers for the experimental results, a standard curve was generated by plating SKOV3.ipl cells in triplicate wells on the day of the assay at the following concentrations: 50,000, 20,000, 10,000, 5,000, 2,000, and 0 cells per well. From the resulting standard curve, cell numbers were calculated for the experimental groups using the SOFTmax computer software (Molecular Devices).

EXAMPLE 7

Human ovarian cancer cell lines allow the transcomplementation of replication-incompetent recombinant adenovirus with replication-enabling plasmids

It has been shown in an animal ovarian carcinoma model that the intraperitoneal administration of an adenoviral vector expressing the HSV-tk gene followed by ganciclovir treatment produced a 50% reduction in tumor mass and a moderate survival advantage. However, there were no complete responses (21). It was hypothesized that this limited effect was based on inadequate tumor cell transduction. Increasing the administered viral inoculum would not represent a feasible option to address this limitation based on reported toxicity (7, 9). As one approach to increase the percentage of cells transduced with a therapeutic gene, it has been shown that tumor cells infected with a replication-defective recombinant adenovirus can be converted into

recombinant adenovirus producing cells by co-transduction of a replication-enabling plasmid (11, 12). An approach based on this strategy for carcinoma of the ovary would require that the transcomplementation physiology were operative in these cells.

Thus, to determine the feasibility of converting ovarian cancer cells to recombinant adenovirus producing cells, a panel of ovarian cancer cell lines were co-transfected with a replication incompetent E1-deleted adenovirus ionically linked to plasmids encoding the adenoviral replication-enabling sequences E1A and E1B. Control groups received virus only or the control plasmid pcDNA3. In addition, HeLa cells, highly transducible by adenovirus-polylysine conjugates, were included in the experiments as a control for the co-transduction efficiency.

Fourty-eight hours after co-transfection, quantifiable adenovirus was present in cell lysates obtained from the co-transduced cells. Log dilutions of lysates were assayed for plaque formation in permissive 293 cells. In all cell lines, lysates from cells co-transduced with the relevant plasmids showed an increase in the titer of adenovirus with respect to cells co-transduced with an control plasmid (Figure 2). The ratios of titers in those groups (mean ± standard deviation of three experiments) were as high as 3,500±1,552, 3,170±859, and 1,000±0 for the PA1, SKOV3.ip1 and OV4 cell lines respectively, and as low as 41±24 and 8±2 for the SW626 and OVCAR-3 cell lines. In contrast, co-transduction with the irrelevant plasmid did not produce, in any case, a viral titer greater than that obtained in cells infected with virus alone. Viral titers determined in cell lysates obtained in the highly transducible HeLa cells were comparable with those reported previously (12) (Figure 2). Thus, the co-transduction of ovarian cancer cells with a

recombinant adenovirus and replication-enabling plasmids increases the viral titer up to three orders of magnitude over that obtained from cells that only received the control plasmid. These experiments establish the feasibility of converting ovarian cancer cell lines into recombinant adenovirus producing cells.

To determine if differences in the production of progeny virus in each ovarian cell line correlated with differences in cell transduction by molecular conjugates, all cell lines were co-transduced in two sets of experiments with adenoviral-polylysine conjugates containing two kinds of reporter gene plasmids expressing the luciferase gene or β-galactosidase. In each experiment, the expression of one reporter gene was read and the other plasmid served as negative control. Expression of the luciferase gene was evaluated at 24 hr post-transduction (Figure 3). Although differences in luciferase expression were observed, indicating variability in cell transduction efficiency, cells showing higher levels of luciferase expression did not necessarily have higher levels of viral output. Staining with X-gal solution of cells co-transduced with the plasmid encoding the β-galactosidase gene showed similar differences in transduction efficiency and lack of correlation with amount of progeny virus (data not shown). Thus, factors other than viral entry and adenoviral transgene expression must play a role in the magnitude of new viral production in each cell line.

EXAMPLE 8

Progeny AdCMVHSV-tk adenovirus is functional and specifically kills

ovarian tumor cells after treatment with ganciclovir

To determine if new virus produced in ovarian tumor cells maintained functionality, killing assays were performed to show cytotoxicity mediated by the virus-encoded toxin gene thymidine kinase in the presence of ganciclovir. Lysates from SKOV3.ip1 cells, which had showed production of a high output of new virus after co-transduction with replication-enabling plasmids, were used to infect fresh cells from the same cell line populations. Given the limited amount of virus available from cell lysates and in order to obtain a quantitative result, the experiment was performed in 96-well plates. To this end, previously titered lysates were used. Infection was performed with a multiplicity of infection (moi) of 1 pfu/cell. Cell survival was determined by means of a colorimetric cell proliferation assay. To control for the cytotoxic effect of recombinant adenovirus itself, a second experimental group was infected with an adenovirus encoding the firefly luciferase reporter gene. Half of the cell samples in both groups were treated with ganciclovir. Cell viability determinations showed that cells infected with SKOV3.ip1 lysates containing progeny AdCMVHSV-tk adenovirus induced a cytotoxic effect when treated with ganciclovir (Figure 4). In contrast, cells infected with AdCMVluc did not show this specific toxicity under ganciclovir treatment. Thus, new virions produced by transcomplementation in this ovarian cancer cell line express the toxin gene thymidine kinase and, therefore, maintain their functionality.

EXAMPLE 9

Transcomplementation physiology can be reproduced in ovarian cancer cells derived from patient ascites

To demonstrate the clinical relevance of this strategy, primary ovarian carcinoma cells were isolated from ascites fluid from women with morphological evidence of ovarian cancer and tested for production of progeny adenovirus in co-transduction experiments. Previous studies examined the efficiency of co-transduction into ovarian cancer primary cells with adenovirus-polylysine and focused more on the percentage of transduced cells rather than the relative efficiency of transduction in different cell types. Thus, a plasmid encoding the β-galactosidase gene was employed. Cells were analyzed 72 hours after co-transduction for expression of the reporter gene. Only 1-5% of the cells were positively stained for β-galactosidase, indicating successful transduction of primary cells although with low efficiency. Notably, over 80% of primary cells infected in parallel with a recombinant adenovirus encoding the same reporter gene were positive for β-galactosidase staining. Thus, transduction of primary cells with adenovirus-polylysine conjugates was feasible, though it was less efficient than that observed in established tumor cell lines.

To determine the feasibility of converting primary ovarian cancer cells to recombinant adenovirus producing cells, a replication incompetent E1-deleted adenovirus was co-transduced with plasmids encoding the adenoviral replication-enabling sequences E1A and E1B into recently isolated primary ovarian cancer cells, as done in previous experiments. Production of progeny virus was again higher in cells transduced with the replication-enabling plasmids than in cells transduced with an irrelevant plasmid (Table 1). Thus, the required physiology for obtaining transcomplementation of

recombinant adenovirus is maintained in primary, patient-derived ovarian cancer cells.

TABLE 1

	Viral titer (pfu/ml)		
	Exp. 1	Exp. 2	Exp. 3
Mock	0	0	0
Virus + pCMVluc4	500	950	350
Virus +pE1A+pE1B	15,000	35,000	35,000

Production of progeny recombinant adenovirus in primary ovarian cancer cells. Viral titers (pfu/ml) were determined by plaque assay of cell lysates obtained 48 hr after co-transduction of cells with AdCMVHSV-tk/pL and the adenoviral replication-enabling sequences E1A and E1B. Control groups received the plasmid pCMVluc4. Results of three experiments are presented.

EXAMPLE 10

Summary

A panel of ovarian cancer cells were co-transfected with a replication-incompetent E1-deleted adenovirus expressing HSV thymidine kinase and plasmids providing E1 sequences needed for replication. Co-transfection was performed using adenovirus-polylysine conjugates. Cells were analyzed for production of new virus by titer determination in growth-permissive 293 cells. The magnitude in the production of progeny recombinant adenovirus was

variable, reaching viral titers three orders of magnitude higher than those obtained by infecting only with the virus. Furthermore, differences in new virus production did not correlate with differences in transduction efficiency, as shown by reporter gene expression.

To determine if new viruses maintain functionality, thymidine kinase-mediated killing assays were performed. Lysates from cell lines showing high virus production were used to infect the same cell line populations. Cell viability determination showed that new virus specifically induced killing in GCV treated cells. A similar viral amplification effect could also be shown in primary ovarian cancer cells derived from patients. The transfection efficiency with molecular conjugates in these cells was low, but did not impede the observation of transcomplementation physiology as measured by progeny virus titration. Thus, transcomplementation strategies have been developed to obtain replication of recombinant adenoviral vectors in ovarian tumor cell lines and in primary ovarian cancer cells. Importantly, the transgene of progeny viruses is fully functional. The present invention establishes the required framework for the *in vivo* co-delivery of adenoviral vectors and replication-enabling plasmids aimed at amplifying the delivery of therapeutic genes. Such a strategy as is described in the instant invention may overcome the limited tumor transduction by current vector systems in clinically relevant contexts.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A viral amplification composition, comprising:

a replication-incompetent, recombinant adenoviral vector, wherein said vector contains a therapeutic gene and at least one gene encoding a replication-enabling function for a non-adenoviral vector; and

a replication-incompetent, non-adenoviral vector, wherein said vector contains at least one gene encoding an adenoviral replication-enabling function, wherein following transduction of a cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions, wherein mutual transcomplementation establishes replication competance resulting in controlled viral amplification in the cell and neighboring cells.

2. The viral amplification composition of claim 1, wherein said recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting of E1, E2 and E4.

3. The viral amplification composition of claim 1, wherein said non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding a viral packaging protein(s).

4. The viral amplification composition of claim

1, wherein said gene encoding said non-adenoviral vector replication-enabling function is a gene encoding a necessary viral packaging protein.

5. The viral amplification composition of claim 1, wherein said gene encoding said adenoviral replication-enabling function is selected from the group consisting of E1A, E1B, E2 and E4.

6. The viral amplification composition of claim 1, wherein said therapeutic gene is selected from the group consisting of the herpes simplex virus thymidine kinase gene, the cytosine deaminase gene and the purine nucleoside phosphorylase gene.

7. The viral amplification composition of claim 1, wherein said non-adenoviral vector is selected from the group consisting of adeno-associated viral vectors and RNA viral vectors.

8. The vector of claim 7, wherein said RNA viral vector is an alphavirus.

9. The vector of claim 7, wherein said RNA viral vector is a Sindbis virus.

10. A method of targeted expression of a therapeutic gene by controlled viral amplification, comprising the step of:

co-transducing a cell with (a) a replication-incompetent,

recombinant adenoviral vector containing a therapeutic gene and at least one gene encoding a replication-enabling function for a non-adenoviral vector; and (b) a replication-incompetent, non-adenoviral vector containing at least one gene encoding adenoviral replication-enabling functions, wherein following transduction of a target cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions and mutual transcomplementation establishes replication competance resulting in controlled viral amplification in the target cell and neighboring cells and the therapeutic gene is expressed in the target cell.

11. The method of claim 10, wherein said recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting of E1, E2 and E4.

12. The method of claim 10, wherein said non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding necessary viral packaging protein(s).

13. The method of claim 10, wherein said genes encoding adenoviral replication-enabling functions are selected from the group consisting of E1A, E1B, E2 and E4.

14. The method of claim 10, wherein said genes

encoding said non-adenoviral vector replication-enabling function is a gene encoding a necessary viral packaging protein.

15. The method of claim 10, wherein said therapeutic gene is selected from the group consisting of the herpes simplex virus thymidine kinase gene, the cytosine deaminase gene and the purine nucleoside phosphorylase gene.

16. The method of claim 10, wherein said cells are selected from the group consisting of ovarian cancer cells and colon cancer cells.

17. A method of killing a tumor cell, comprising the steps of:

(1) co-transducing said tumor cell with (a) a replication-incompetent, recombinant adenoviral vector containing the herpes simplex virus thymidine kinase gene and at least one gene encoding replication-enabling functions for a non-adenoviral vector, and (b) a replication-incompetent, non-adenoviral vector containing at least one gene encoding adenoviral replication-enabling functions, wherein following transduction of the tumor cell by the adenoviral vector and the non-adenoviral vector, mutual transcomplementation of the vectors occurs whereby each vector provides replication enabling function to the other vector by virtue of expressing the genes encoding replication-enabling functions establishing replication competance resulting in controlled viral amplification in the tumor cell and neighboring cells; and

(2) treating said co- transduced tumor cells with

ganciclovir, wherein ganciclovir is converted to a toxin by the product of said thymidine kinase gene resulting in tumor cell cytotoxicity.

18. The method of claim 17, wherein said recombinant adenoviral vector is replication-incompetent due to a deletion in at least one gene selected from the group consisting of E1, E2 and E4.

19. The method of claim 17, wherein said non-adenoviral vector is replication-incompetent due to deletion of gene(s) encoding a viral packaging protein(s).

20. The method of claim 17, wherein said gene encoding said non-adenoviral vector replication-enabling function is a gene encoding a necessary viral packaging protein.

21. The method of claim 17, wherein said gene encoding said adenoviral replication-enabling function is selected from the group consisting of E1A, E1B, E2 and E4.

22. The method of claim 17, wherein said non-adenoviral vector is selected from the group consisting of adeno-associated viral vectors and RNA viral vectors.

23. The method of claim 17, wherein said tumor cells are selected from the group consisting of ovarian cancer cells

and colon cancer cells.

E1-deleted adenovirus
(replication-incompetent DNA virus)
Structural genes-deleted Sindbis
(replication-incompetent RNA virus)
Recombinant Adenovirus
that will deliver DNA
encoding structural
proteins of Sindbis
Recombinant Sindbis
virus that will
deliver RNA encoding
E1A/E1B of adenovirus
Adenovirus gets its
E1A/E1B;thus it
replicates, kills
the cell, and spreads
to nearby cells
Sindbis gets its
structural proteins;
thus it replicates and
spreads to nearby cells

FIGURE 1

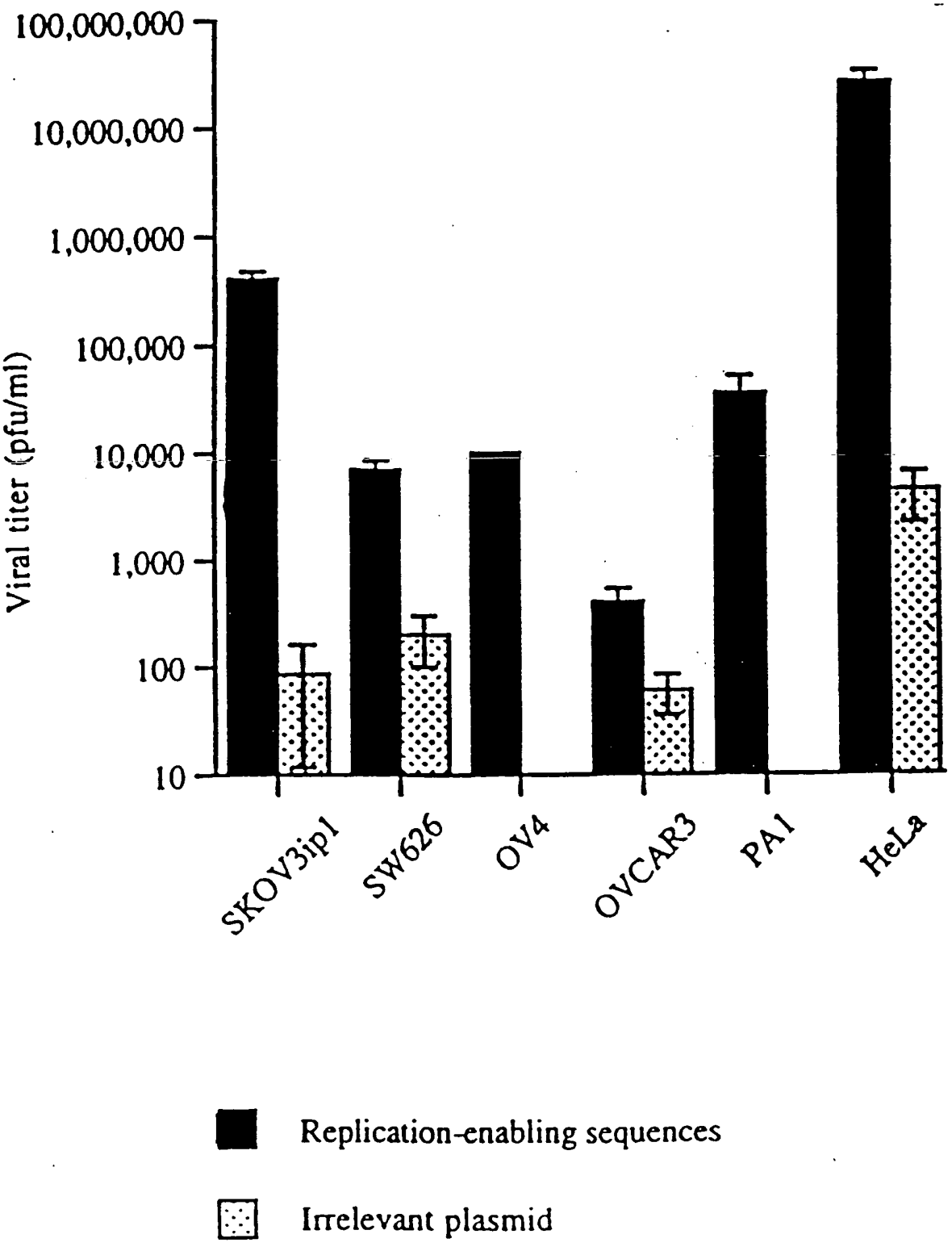
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
Viral titer (pfu/ml)
SKOV3ip1
SW626
OV4
OVCAR3
PA1
HeLa
Replication-enabling sequences
Irrelevant plasmid

FIGURE 2

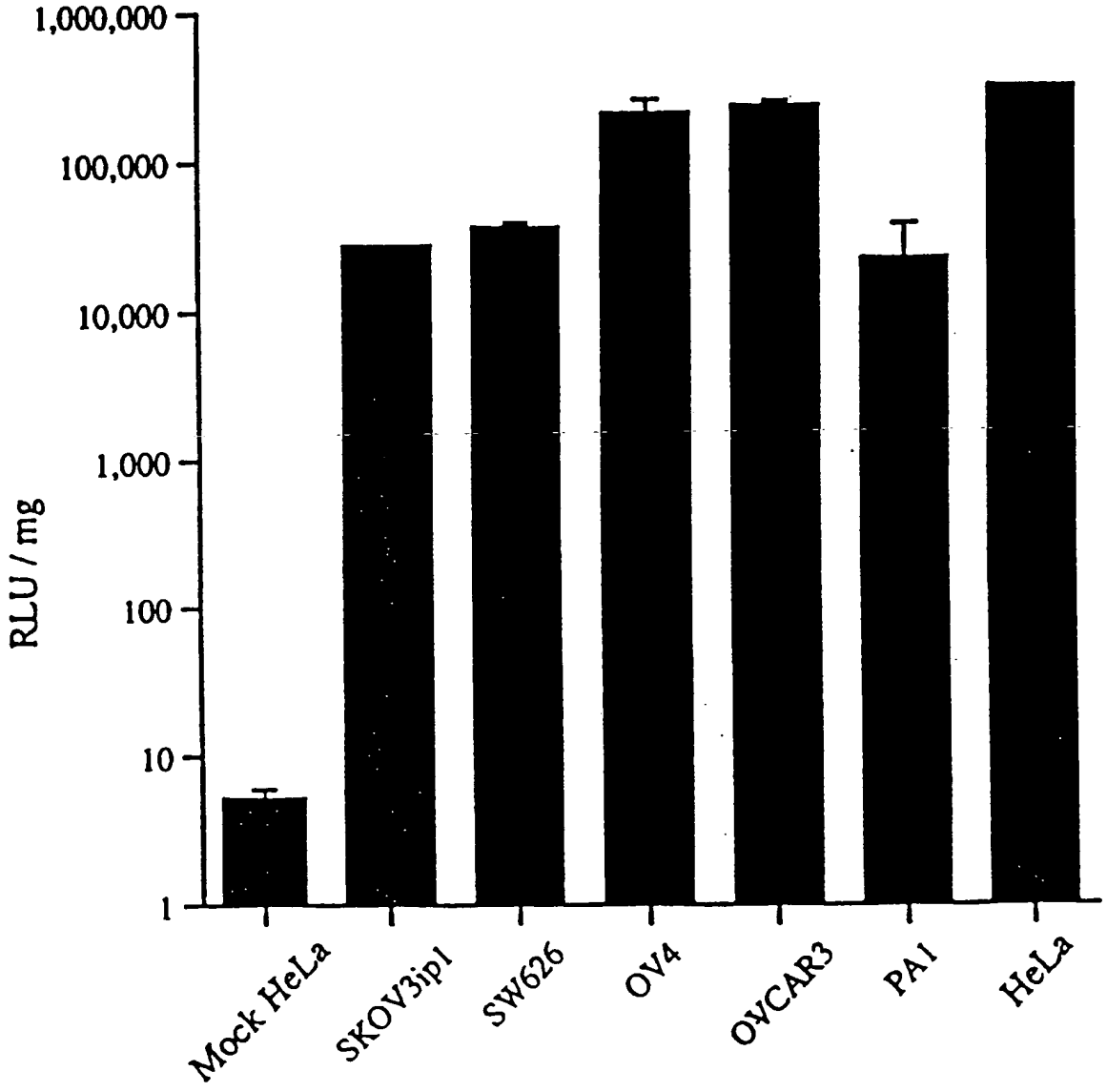
1,000,000
100,000
10,000
1,000
100
10
1
RLU / mg
Mock HeLa
SKOV3ip1
SW626
OV4
OVCAR3
PA1
HeLa

FIGURE 3

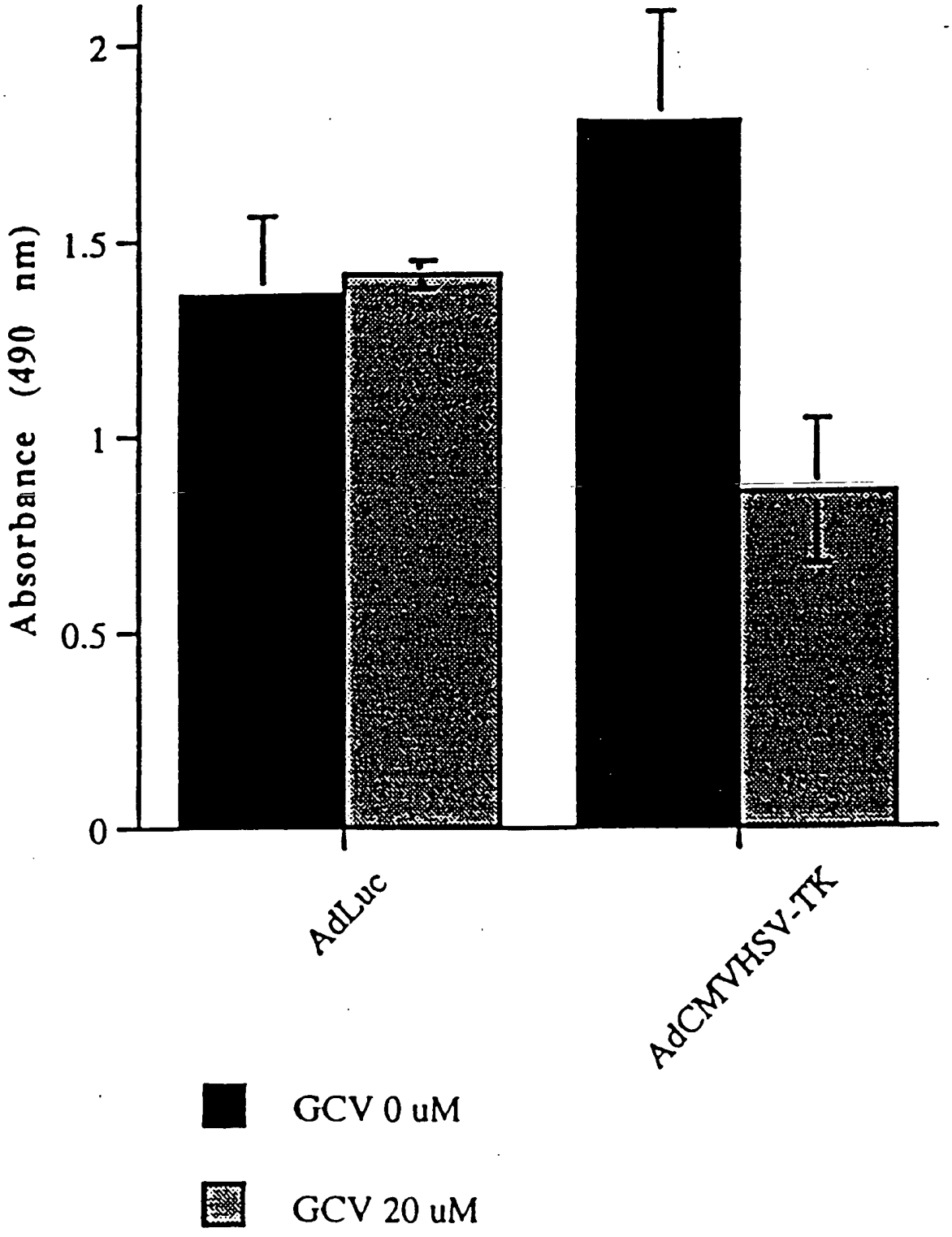
Absorbance (490 nm)
2
1.5
1
0.5
0
AdLuc
AdCMVHSV-TK
GCV 0 uM
GCV 20 uM

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04394

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 43/04; C12N 15/00;
US CL :435/320.1, 440, 455, 456, 457; 514/44;
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 440, 455, 456, 457; 514/44;

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	WO 98/22143 A1 (UNIVERSITY OF ALABAMA AT BIRMINGHAM RESEARCH FOUNDATION) 28 MAY 1998, see throughout.	1-23
A,P	DUISIT et al. Functional Characterization of Adenoviral/Retroviral Chimeric Vectors and Their Use for Efficient Screening of Retroviral Producer Cell Lines. Human Gene Therapy. 20 January 1999, Vol. 10, pages 189-200, see entire document.	1-23
A,P	RAMSEY et al. Adenovirus Vectors as Transcomplementing Templates for the Production of Replication Defective Retroviral Vectors. Biochemical and Biophysical Research Communications. 29 May 1998, Vol. 246, No. 3, pages 912-919, see entire document.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
21 MAY 1999	02 JUN 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DEBORAH J. R. CLARK
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04394

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS
search terms: adenovirus, retrovirus, packaging, incompetent, defective, deficient, transcomplementation, complementation, in trans